

The Effect of Dose, Dose Rate, Route of Administration, and Species on Tissue and Blood Levels of Benzene Metabolites

by Rogene F. Henderson,* Patrick J. Sabourin,*
William E. Bechtold,* William C. Griffith,*
Michele A. Medinsky,* Linda S. Birnbaum,[†] and
George W. Lucier[†]

Studies were completed in F344/N rats and B6C3F₁ mice to determine the effect of dose, dose rate, route of administration, and rodent species on formation of total and individual benzene metabolites. Oral doses of 50 mg/kg or higher saturated the capacity for benzene metabolism in both rats and mice, resulting in an increased proportion of the administered dose being exhaled as benzene. The saturating air concentration for benzene metabolism during 6-hr exposures was between 130 and 900 ppm. At the highest exposure concentration, rats exhaled approximately half of the internal dose retained at the end of the 6-hr exposure as benzene; mice exhaled only 15% as benzene. Mice were able to convert more of the inhaled benzene to metabolites than were rats. In addition, mice metabolized more of the benzene by pathways leading to the putative toxic metabolites, benzoquinone and muconaldehyde, than did rats. In both rats and mice, the effect of increasing dose, administered orally or by inhalation, was to increase the proportion of the total metabolites that were the products of detoxification pathways relative to the products of pathways leading to putative toxic metabolites. This indicates low-affinity, high-capacity pathways for detoxification and high-affinity, low-capacity pathways leading to putative toxic metabolites. If the results of rodent studies performed at high doses were used to assess the health risk at low-dose exposures to benzene, the toxicity of benzene would be underestimated.

Introduction

Problem

In the absence of dose-response data in humans, long-term rodent studies involving hundreds of animals are used to predict the carcinogenicity of compounds for people. However, the responses of rodents to compounds may vary widely between species, both in the type of response and in the amount of compound eliciting a response. Also, for convenience or for economic reasons, rodent studies may not use the same route of exposure or the same dose rate as that expected in humans. To be able to interpret these animal data for extrapolation to

health risk assessments for humans, one must obtain more information on the basis for the rodent responses. Important areas to consider are the effect of species, route of exposure, and dosing regimen on the disposition and metabolic fate of the administered material. Such information is critical for determining the biologically effective dose delivered to target sites in the body under the different exposure situations. The following is a report of studies conducted at the Lovelace Inhalation Toxicology Research Institute in collaboration with the National Institute of Environmental Health Sciences to determine the disposition and metabolic fate of benzene in rats and mice under different exposure conditions.

Background

The major concern in chronic exposures of humans to benzene is its adverse hemopoietic effects and neoplastic or preneoplastic effects in other tissues. The incidence of leukemia, pancytopenia, and preleukemia in humans exposed to benzene is significantly higher than in the

*Inhalation Toxicology Research Institute, Lovelace Biomedical and Environmental Research Institute, P. O. Box 5890, Albuquerque, NM 87185.

[†]National Institute of Environmental Health Sciences, P. O. Box 12233, Research Triangle Park, NC 27709.

Address reprint requests to R. F. Henderson, Inhalation Toxicology Research Institute, Lovelace Biomedical and Environmental Research Institute, P.O. Box 5890, Albuquerque, NM 87185.

general population (1). Benzene may also be a factor in the development of other forms of cancer, such as Hodgkin's disease, multiple myeloma, and lung cancer (1).

Studies by Maltoni et al. (2,3) and by the National Toxicology Program (NTP) (4) clearly show that benzene administered to rodents by gavage or inhalation produces neoplasia in a variety of tissues. In the NTP studies B6C3F₁ mice and female F344/N rats received 0, 25, 50, 100 mg benzene/kg orally, in corn oil, 5 days/week for 103 weeks. Male F344/N rats received 0, 50, 100, and 200 mg benzene/kg in the same dosing regimen. Maltoni and co-workers exposed Sprague-Dawley rats to 200 to 300 ppm benzene during a 2-year period and also conducted an oral study in which Sprague-Dawley rats were administered 0, 50, and 250 mg benzene/kg in olive oil, 5 times/week for 52 weeks and were held for 92 weeks for observation. The tumorigenic responses in these studies were species-dependent. Mice developed neoplasms in the lung, Harderian gland, preputial gland, ovary, mammary gland, and liver while rats did not. Benzene induced neoplasms in the Zymbal gland of both rats and mice and in the oral cavity and skin of rats only. To interpret the results of such animal studies for human risk assessment, it is important to know the effect of species, route of administration, total internal dose, and rate at which the dose was delivered on the disposition and metabolic fate of the benzene.

Approach

Two types of studies were conducted to address the problem described. First, ¹⁴C-benzene was used to determine the effect of species, exposure dose, and route of exposure on the excretion patterns and total metabolites formed from benzene. ("Exposure dose" is used here to indicate the amount of material presented to the animal by any route of exposure, i.e., the amount orally instilled, the amount injected, IP, or the inhalation exposure concentration over a specific time.) Second, the effect of species, exposure dose, exposure dose rate, and the route of exposure on tissue and blood levels of individual metabolites was determined using ³H-benzene. The ³H-benzene was required to obtain a high enough specific activity in benzene metabolites to be able to detect them in tissues. Because the individual metabolites were isolated prior to radioactivity determination, there was not a problem with measuring tritium that was not associated with the metabolites. Initial studies using both ¹⁴C- and ³H-labeled benzene indicated only a minor (~10%) isotope effect for any of the metabolites (7).

Methods

The methods for the conduct of the studies have been published (5-8). Inhalation exposures were in nose-only chambers described by Raabe et al. (9). Analysis of organic-soluble metabolites of benzene (7) was performed on ethyl acetate extracts of samples to which excess (carrier) unlabeled benzene and its metabolites had been

added. Butylated hydroxytoluene was added as an antioxidant. The metabolites were separated by semi-preparative reverse-phase high-performance liquid chromatography (HPLC). Isolated compounds were analyzed for radioactivity (by liquid scintillation spectrometry) and for mass (by UV absorption). The total amount of each compound present was calculated from the mass dilution of the radiolabeled isotope. An ion-pairing HPLC method was developed to analyze the water-soluble metabolites (8). Ascorbate was added as an antioxidant to the water-soluble fraction of tissue extracts and +D-saccharic acid-1,4-lactone was added to inhibit the breakdown of glucuronide conjugates. The metabolites were separated by reverse-phase HPLC using tetrabutyl ammonium hydrogen sulfate as the ion-pairing agent. In calculating the amount of both water-soluble and organic-soluble benzene metabolites, the expected loss of ³H during metabolism was taken into account.

Excretion Routes and Total Benzene Metabolites Formed

This study has been reported in detail (5). ¹⁴C-Benzene was administered to the species used in the long-term bioassay studies (F344/N rats, Sprague-Dawley rats, and B6C3F₁ mice) orally, by IP injection, and by inhalation. In the inhalation studies, the internal dose retained in the animals was determined at the end of the 6-hr exposure by liquid scintillation spectroscopy. Routes of excretion were determined by measuring ¹⁴C in urine, feces, and exhaled air (separating CO₂ from benzene) for 48 hr after dose administration. Total metabolites were determined by summing the ¹⁴C-activity in urine, feces, CO₂, and remaining in the carcass. Mass balance between the administered dose and the total amount excreted or retained in the body was determined and indicated recoveries of 100 ± 12% ($\bar{x} \pm SD$, $n = 19$ studies) (5).

By any route of exposure, over 95% of the administered radioactivity had been excreted in 48 hr, and approximately 90% of all metabolites were excreted in the urine. In both rats and mice, there was an increased amount of the administered dose that was exhaled as benzene at doses of 50 mg/kg or higher (Fig. 1). [There were no differences in the results observed in the two strains of rats (5); therefore, the rat data have been combined.] The same excretion patterns were observed after IP injection (Fig. 2). Concomitant with the increased amount of exhaled benzene at the higher doses, there was a decrease in the percent of the dose converted to metabolites.

The results from the inhalation study are shown in Table 1. The mice received a higher internal dose for the equivalent exposure conditions than did the rats. The mice were also better able to metabolize the inhaled benzene at the higher concentration and exhaled less benzene after exposure. Why do mice receive a higher internal dose than rats in the inhalation exposures? Mice have a higher minute volume per kilogram body weight than rats (~1.5 ×), which would cause the blood concentrations to reach equilibrium more quickly than in rats but

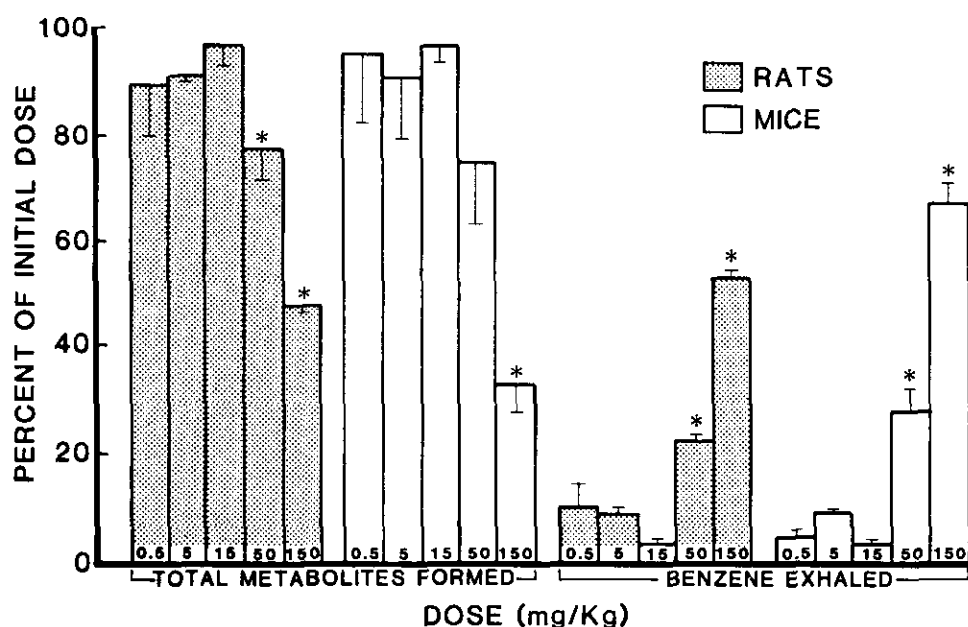


FIGURE 1. Total metabolites formed from orally administered benzene in F344/N rats and B6C3F₁ mice during a 48-hr period after dosing. Total metabolites were calculated as the sum of ¹⁴C in urine, feces, exhaled CO₂, and ¹⁴C remaining in carcass at end of 48 hr. The administered dose, in milligrams per kilogram, is shown at the bottom of each bar. Bars represent the mean \pm SE of values from three animals (5). An asterisk (*) indicates means that differ from values at doses less than 50 mg/kg, $p \leq 0.05$.

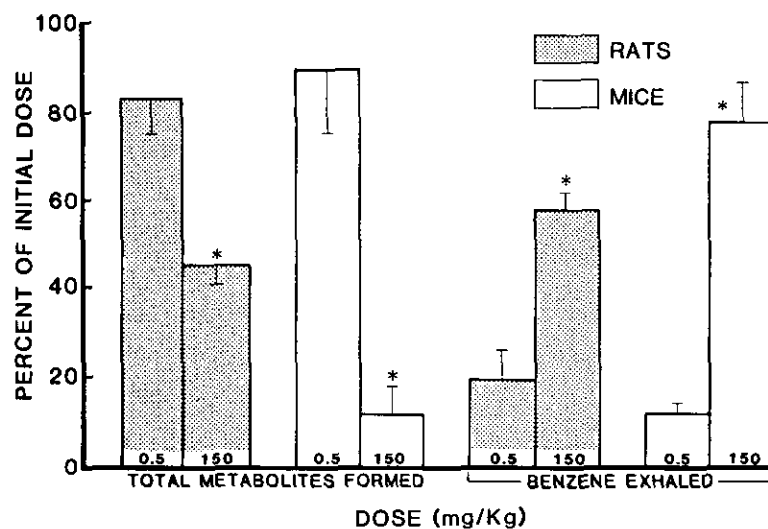


FIGURE 2. Total metabolites formed from IP-administered benzene in F344/N rats and B6C3F₁ mice during a 48-hr period after dosing. Total metabolites were calculated as the sum of ¹⁴C in urine, feces, exhaled CO₂, and ¹⁴C remaining in carcass at end of 48 hr. The administered dose, in milligrams per kilogram, is shown at the bottom of each bar. Bars represent the mean \pm SE of values from three animals (5). An asterisk (*) indicates means that differ from values at doses less than 50 mg/kg, $p \leq 0.05$.

Table 1. Metabolite formation from inhaled benzene.^a

Exposure concentration, ppm	Internal dose, mg/kg ^b		% of initial dose			
			Total metabolites		Exhaled benzene	
	Rats	Mice	Rats	Mice	Rats	Mice
11	3.3 \pm 0.3	7.5 \pm 1.1	96 \pm 1	99 \pm 2	4 \pm 0.1	0.1 \pm 0.1
130	24 \pm 2	60 \pm 6	95 \pm 1	99 \pm 1	5 \pm 0.5	1 \pm 0.2
930 ^c	117 \pm 40	152 \pm 14	52 \pm 2	86 \pm 5	48 \pm 2	14 \pm 4

^aBased on excreta collections over a 48-hr period after a 6-hr exposure. Values are means \pm SE, $n = 3-5$.

^bBased on ¹⁴C-benzene equivalents in body of rodents at end of 6-hr exposure.

^cAverage of actual exposure concentration for mice (990 ppm) and rats (870 ppm).

would not affect the steady-state level in blood. The rate of removal of benzene from the blood would depend on metabolism, and a higher rate of metabolism in the mouse would account for both the high internal dose at all exposure concentrations and the decreased exhalation of benzene at the highest exposure. In mice, it is possible to achieve higher internal doses by inhalation than by the oral or IP route (compare Table 1 with Figs. 1 and 2). For example, the highest 6-hr inhalation exposure in mice resulted in an internal dose at the end of the exposure of 152 mg/kg. Of this, 86% or 130 mg/kg, was converted to metabolites. By contrast, oral or IP doses of 150 mg to mice resulted in only 20 to 30% conversion of the dose to metabolites, with the remainder being exhaled as benzene. Because of the rapid exhalation of the bolus doses given IP or orally in mice, only limited internal doses can be attained by these routes. However, administration of benzene by inhalation over a 6-hr period allows time for mice to metabolize the benzene. Thus, when one compares an oral dose to the equivalent dose by inhalation in terms of total metabolites formed (Table 2), one finds that it is difficult to achieve as high a level of benzene metabolites in a mouse by a single oral dose as one can achieve by inhalation.

In summary, this study indicated a) benzene is rapidly metabolized and excreted, mainly as water-soluble metabolites in urine, within 40 hr of dosing by any route; b) bolus doses of 50 mg/kg or higher exceed the ability of rodents to metabolize benzene and part of the dose will be lost by exhalation of benzene; c) mice metabolize benzene more rapidly than rats; and d) benzene administered at a slow dose rate, as in an extended (e.g., 6 hr) inhalation exposure, will result in higher internal doses in mice than bolus dosing. According to these data, almost all the oral doses given in the long-term bioassay studies described in the background section of this paper were beyond the linear range for formation of metabolites in the rodents. Because the high doses are not converted to metabolites as efficiently as lower doses, the use of the

bioassay data to predict the effects of low doses of benzene may underestimate the toxicity of the compound.

Benzene Metabolites

The next part of our study was to determine the effect of species and dosing regimen on the amount of individual benzene metabolites in blood and tissues because our information on total metabolites formed did not distinguish between toxic and nontoxic metabolites. The major metabolic pathways of benzene (4) are illustrated in Figure 3. There are several detoxification pathways. One is the formation of glutathione conjugates of benzene oxide leading to formation of prephenyl mercapturic and phenyl mercapturic acid. A second is the formation of glucuronide or sulfate conjugates of phenol. Two metabolic pathways form putative toxic metabolites, muconaldehyde (10) and benzoquinone (11). In our analyses, we have used stable compounds at the end or close to the end of these pathways as markers for the amount of the benzene being metabolized by the different pathways (Table 3). In all blood or tissue samples analyzed, greater than 90% of metabolites were in water-soluble forms (Table 4). Therefore, major emphasis was placed on quantitating the water-soluble benzene metabolites as indicative of benzene metabolism through the major pathways.

Species Differences in Benzene Metabolism

This study has been reported in detail previously (6). F344/N rats and B6C3F₁ mice were exposed by inhalation to 50 ppm ³H-benzene for 6 hr. Tissue and blood samples were analyzed for benzene and its metabolites both during and for 8 hr after the exposure and the metabolite concentration plotted versus time. The integrated dose to a tissue over the 14-hr period (6 hr of exposure, 8 hr following exposure) was calculated for each metabolite (area under curve or AUC). The results are shown in Table 4.

The major metabolic products in rats were detoxification products—phenyl conjugates. Hydroquinone and its conjugates, hydroquinone glucuronide and hydroquinone sulfate, were either not detectable in rats or present in trace quantities. On the other hand, mice had substantial quantities of the markers for the toxification pathways (muconic acid, hydroquinone glucuronide, and hydroquinone sulfate) in tissues in addition to the markers for the detoxification pathways. Bone marrow samples taken at different times were pooled to have sufficient radioactivity for analysis (Fig. 4). The only metabolite detected in rat bone marrow was phenyl sulfate. In mouse bone marrow, muconic acid and hydroquinone glucuronide were also detected. Thus, not only do mice metabolize benzene faster than rats, mice also form more of the putative toxic metabolites than do rats.

Table 2. Extrapolation of oral dose to equivalent 6-hr inhalation dose.

6-hr inhalation exposure, ppm	Total metabolites formed, mg/kg		Equivalent single oral dose, mg/kg	
	Rats	Mice	Rats	Mice
10	3	10	3	12
25	6	25	6	40
50	12	50	12	150
100	25	80	30	— ^a
200	40	110	80	—
600	80	150	200	—

^aHigher oral doses in mice are mostly exhaled as benzene (Fig. 1); experimentally, we did not achieve total metabolite levels higher than 50 mg/kg in mice by a single oral dose. According to a mathematical model based on these data, a single oral dose of greater than 300 mg/kg would be required to achieve 80 mg/kg benzene metabolites in mice.

METABOLIC SCHEME FOR BENZENE

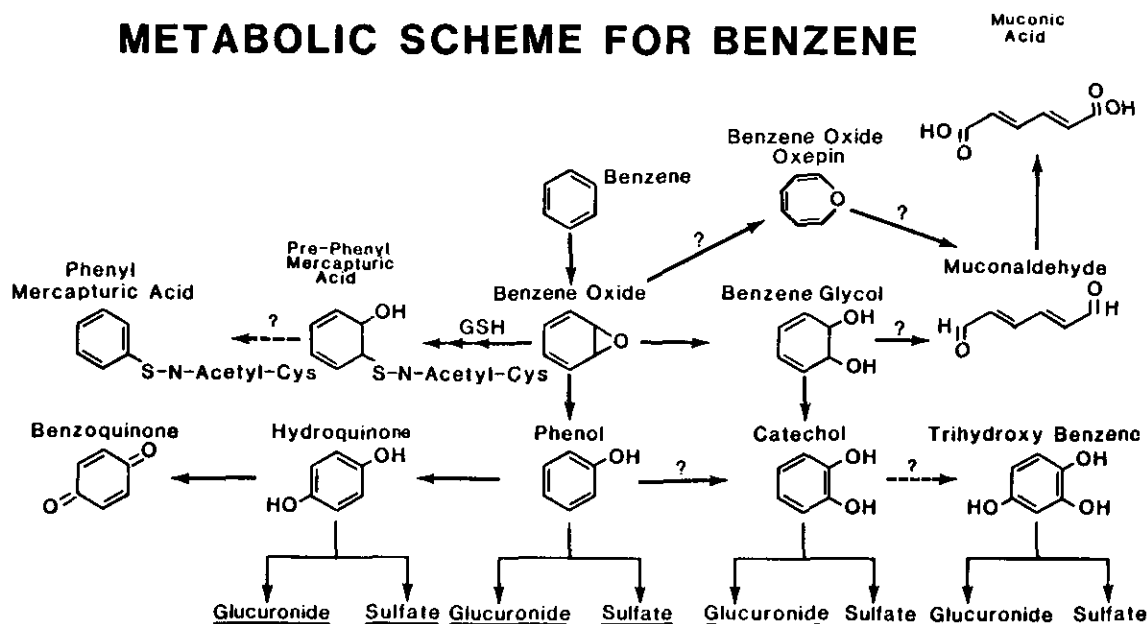


FIGURE 3. Metabolism of benzene. Modified from Huff et al. (4).

Table 3. Markers of benzene metabolism.

Pathways	Markers
Toxicification	
Pathway leading to ring breakage (muconaldehyde)	Muconic acid
Pathway leading to benzoquinone	Hydroquinone glucuronide or hydroquinone
Detoxification	
Pathway leading to mercapturic acid products	Prephenyl mercapturic acid Phenyl mercapturic acid
Pathways leading to phenyl conjugates	Phenyl sulfate Phenyl glucuronide

Effect of Exposure Dose on Formation of Benzene Metabolites

This study has been reported in detail elsewhere (12). F344/N rats and B6C3F₁ mice were exposed orally to 1, 10, and 200 mg ³H-benzene/kg body weight or by inhalation to 5, 50, or 600 ppm ³H-benzene for 6 hr. The highest oral dose and exposure concentration were expected to be outside the linear range for total metabolism of benzene as determined by earlier studies. ("Linear range" refers to a linear relationship between exposure dose and

Table 4. Metabolites formed after a 6-hr inhalation exposure to 50 ppm benzene.^a

	Rats			Mice		
	Liver	Lung	Blood	Liver	Lung	Blood
Organic-soluble metabolites						
Phenol	0.4 ± 0.6	0.2 ± 0.3	ND	1.8 ± 0.5	2.7 ± 0.8	2.6 ± 2.0
Catechol	0.03 ± 0.14	0.02 ± 0.09	ND	5.5 ± 4.0	0.8 ± 0.4	ND
Hydroquinone	0.07 ± 0.12	ND	0.3 ± 0.3	15 ± 3.0	6.1 ± 1.5	8.0 ± 7.2
Water soluble metabolites						
Phenyl sulfate	94 ± 29	113 ± 15	136 ± 26	145 ± 29	231 ± 37	228 ± 49
Phenyl glucuronide	ND	ND	ND	28 ± 15	9.9 ± 3.3	2.7 ± 2.2
Hydroquinone glucuronide	ND	ND	ND	189 ± 27	113 ± 17	105 ± 14
Muconic acid	66 ± 10	14 ± 3	4.6 ± 2.6	1220 ± 96	110 ± 13	7.9 ± 5.3
Phenyl and prephenyl mercapturic acid	36 ± 7	4.9 ± 1.8	0.9 ± 0.7	223 ± 31	11 ± 2.6	14 ± 20

^aValues are areas under the curve for metabolite concentrations in the blood and tissue samples over a 14-hr period (6 hr of exposure and 8 hr following exposure). Values are means ± SE in nmole·hr/g, n = 4. ND, not detected; for limits of detection in each tissue, see Bechtold et al. (7) and Sabourin et al. (8).

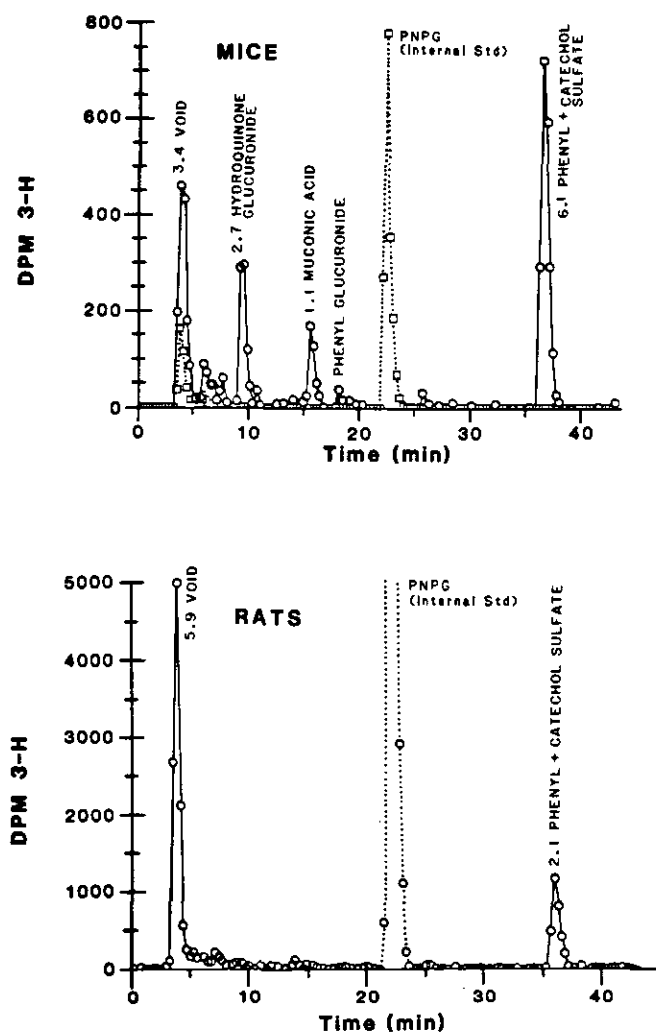


FIGURE 4. Water-soluble benzene metabolites in pooled bone marrow samples taken during and for 8 hr following a 6-hr 50-ppm ^3H -benzene exposure (6). Bone marrow samples taken at various time points were pooled and processed and analyzed by HPLC as described in "Methods." The HPLC chromatogram is shown. Numbers above each peak refer to the nmole metabolite per gram of bone marrow tissue. The internal standard was *p*-nitrophenyl glucuronide (PNPG).

the amount of total benzene metabolites formed.) It was not known if these exposure levels would be outside the linear range for the amount of individual metabolites formed. The amount of individual benzene metabolites formed per unit of exposure was determined in blood and major tissues. Representative results for a marker of the major detoxification pathway (phenyl sulfate) and a marker of a toxification pathway (muconic acid) in the blood are illustrated in Figure 5 for inhaled benzene and in Figure 6 for orally administered benzene. The graphs are normalized to the exposure dose (ppm for inhalation; mg/kg for oral route) and the curves should be superimposed on one another if the formation of the metabolite is linearly related to exposure dose. The amount of the major metabolite, phenyl sulfate, formed from inhaled

benzene (Fig. 5) was linear with respect to exposure dose at 600 ppm in mice but not in rats. (In mice, the curve was shifted to the right in the 600-ppm exposure, but the AUC/ppm was not different from that of the two lower exposure concentrations.) In contrast, the amount of muconic acid formed was nonlinear after all inhalation exposure concentrations in both species, with proportionally (as percent of the exposure concentration) more muconic acid formed at the lower exposure concentration. If muconic acid is involved in the toxicity from inhaled benzene, one would underestimate the toxicity of the compound if one extrapolated from the effects at high doses in animal studies to expected results at low doses.

Following oral exposures of rats, the blood concentrations of both phenyl sulfate and muconic acid were nonlinearly related to dose at the highest exposure dose, indicating saturation of both pathways of metabolism. In mice there was a saturation of the pathway leading to muconic acid formation but a trend toward increased formation of the detoxification metabolite, phenyl sulfate.

In addition, an increasing proportion of the inhaled benzene was converted to glutathione conjugates and to phenyl glucuronide at the higher exposure concentration in mice (12). Thus, in mice, there appeared to be a shift toward detoxification pathways at the higher exposure dose. Hydroquinone conjugates were barely detectable in rats but decreased in proportion relative to the exposure concentration in mice in a manner similar to that observed for muconic acid. The same trends toward increased formation of glutathione conjugates and phenyl glucuronide and decreased formation of hydroquinone conjugates at the highest dose were noted in orally exposed mice.

In summary, the effect of increasing dose, administered to rats or mice either orally or by inhalation, was to increase the proportion of markers of detoxification pathways relative to the markers of pathways leading to putative toxic metabolites.

Effect of Exposure Dose Rate on Metabolic Fate of Benzene

Because previous studies indicated differences between a bolus dose (given orally) and a dose administered over 6 hr (by inhalation) (5), a study was designed to determine the effect on benzene metabolism of extended inhalation exposures to low concentrations versus short exposures to high levels (12). Fischer 344/N rats and B6C3F₁ mice were exposed by inhalation to one of three exposure regimens, all having the same concentration \times time factor: 600 ppm benzene for 0.5 hr, 150 ppm for 2 hr, or 50 ppm for 6 hr. The AUCs for benzene metabolites in blood and tissues were determined. If there were no dose rate effects, the ACUs should be the same for all exposure regimens. In general, there was no dose-rate effect in rats. In mice, however, the fast exposure rate (0.5 hr \times 600 ppm) produced less muconic acid in blood, liver, and lung (Fig. 7). In the blood and lung, less hydroquinone

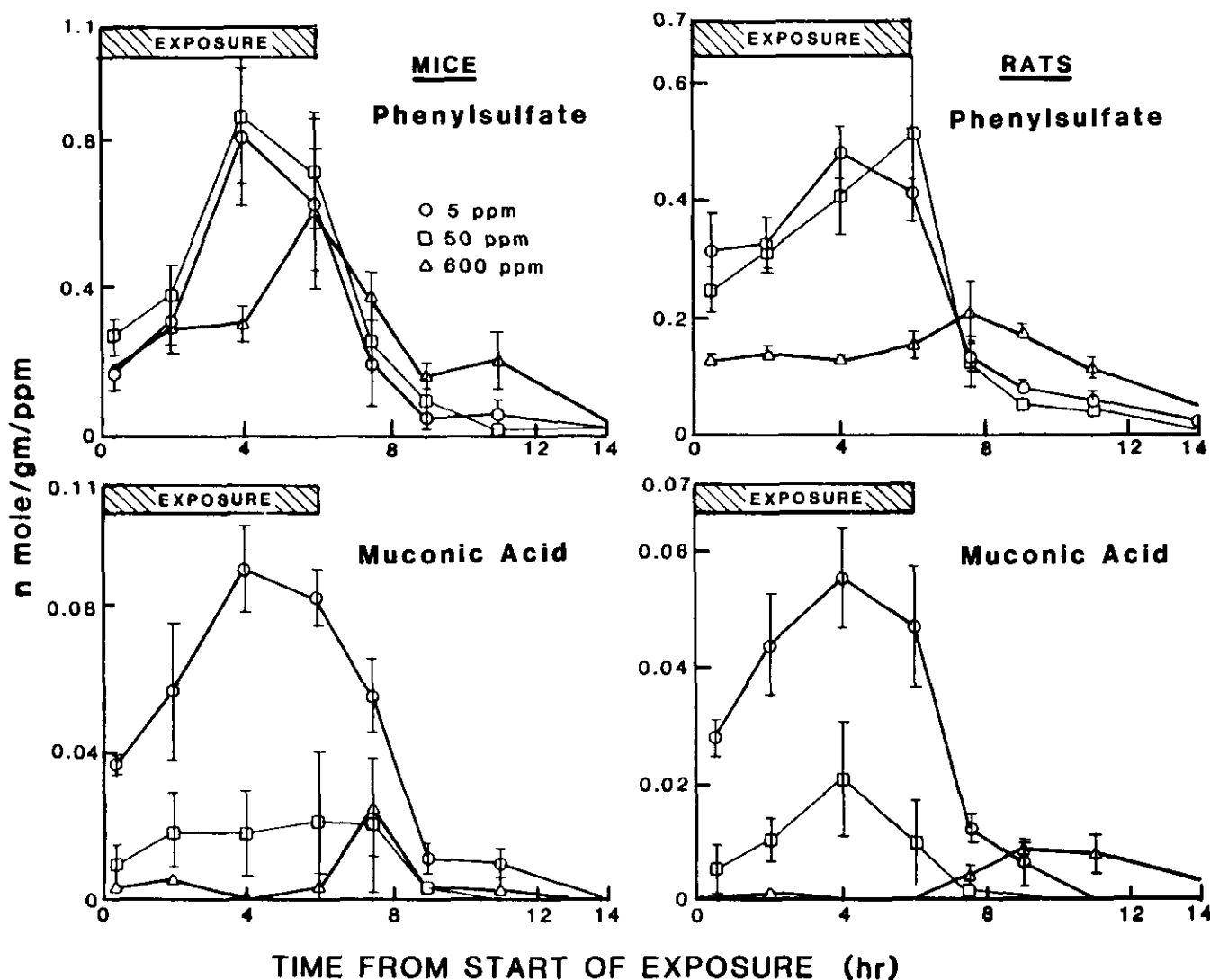


FIGURE 5. Blood metabolites during and after exposure of F344/N rats and B6C3F₁ mice to 5, 50, or 600 ppm benzene for 6 hr. The nanomoles metabolite per gram of blood has been normalized to the part per million exposure to allow comparison of the effect of exposure concentration on the amount of metabolites produced. If the increase in metabolite concentration is linearly related to the increase in exposure concentration, the curves will be superimposed on each other. Points and bars represent the mean \pm SE of four values. This illustration is from an earlier publication (12).

glucuronide was produced at the high exposure rate, and more prephenyl mercapturic acid was produced at the two higher dose rates. In the pooled bone marrow samples (data not shown), there was a reduction in the ratios of the markers of the toxic benzene metabolic pathways (muconic acid and hydroquinone glucuronide) to the major metabolite (phenyl sulfate) at the highest exposure rate in mice. These results are similar to what was observed in mice exposed to 600 ppm for 6 hr and confirm the fact that at high exposure concentrations, mice tend to shift a greater portion of their benzene metabolism toward detoxification pathways. In summary, the effect of

increased dose rate in inhalation exposures of mice appears to be the same as for increased exposure concentrations.

Summary

The results of these studies indicate mice metabolize benzene faster than rats and convert more of the benzene to toxic metabolites that do rats. For both species, in general, the detoxification pathways for benzene appear to be low-affinity, high-capacity pathways, whereas pathways leading to the putative toxic metabolites appear to

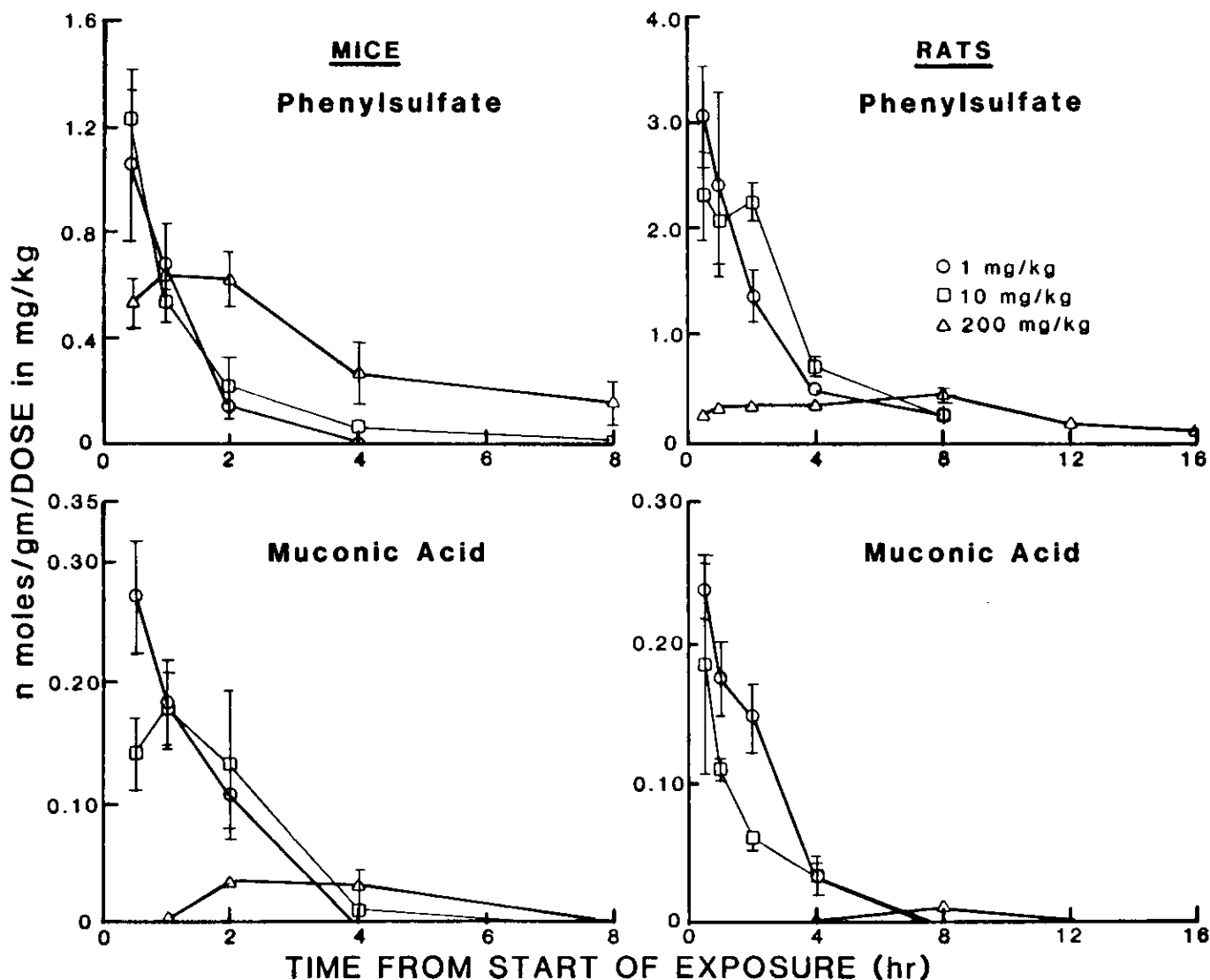


FIGURE 6. Blood metabolites following an oral exposure of F344/N rats and B6C3F₁ mice to 1, 10, or 200 mg/kg benzene in corn oil. The nanomoles metabolite per gram of blood has been normalized to the dose in milligrams per kilogram to allow comparison of the effect of dose on the amount of metabolite produced. If the increase in metabolite concentration is linearly related to the increase in dose, the curves will be superimposed on each other. Points and bars represent the mean \pm SE of four values. This illustration is from an earlier publication (12).

be high-affinity, low-capacity systems. The net result of extending exposure dose regimens beyond the range of linear metabolism rates (above ~ 200 ppm by inhalation or ~ 50 mg/kg by the oral route) is to reduce the amount of toxic metabolites formed relative to the amount of administered material. In a risk assessment process, if the results of animal studies performed at high exposure doses were extrapolated to assess the health risk to humans exposed to low doses of benzene, the toxicity of benzene would be underestimated.

The major objective of these studies was to obtain information to allow better extrapolation of the results of animal studies to expected results in humans. To achieve,

this, the animal data have been used to develop a mathematical model to predict the disposition and metabolic fate of benzene under all exposure conditions in rodents (13,14). The next step is to alter the physiological parameters used in this model to fit the known physiological values for humans. The model can also be strengthened by making use of known values for metabolism of benzene by human tissues *in vitro*. Finally, limited data from humans exposed accidentally to benzene can be used to validate the model. With this approach, the animal toxicokinetic data developed in these studies should prove useful in improving our ability to assess human health risks associated with benzene exposures.

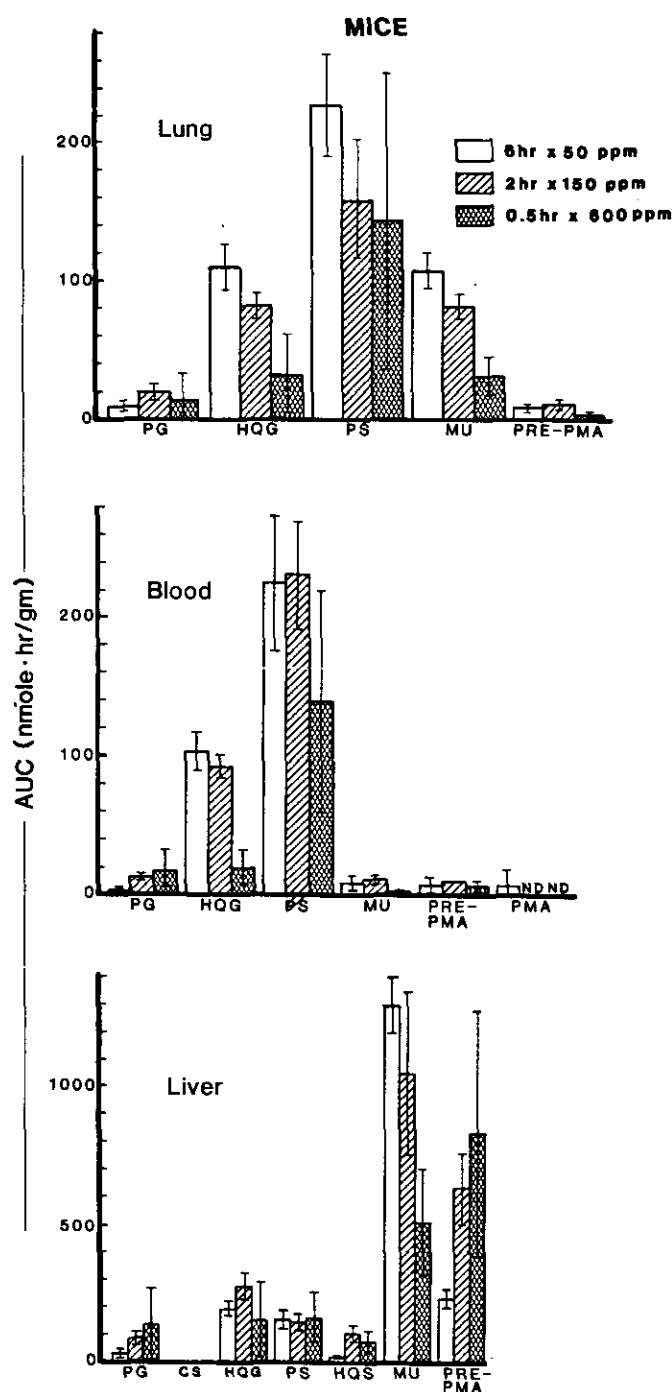


FIGURE 7. The effect of exposure rate on lung, liver, and blood metabolites formed in B6C3F₁ mice exposed to benzene by inhalation. The mice were exposed by the indicated exposure regimens which have the same concentration times time factor ($C \times T = 300$ ppm/hr). The tissue or blood metabolite concentrations were determined at regular intervals during and after the exposures and were plotted versus time after initiation of the exposures. The area under the curve (AUC), which represents the dose of the metabolite received by the tissue, is shown for each metabolite for each dose rate. If the dose rate does not influence the tissue metabolite dose, the bars (AUCs) will be equal for all dose rates. Values shown are the mean \pm SE of four samples. PG, phenyl glucuronide; HQG, hydroquinone glucuronide; PS, phenyl sulfate; MU, muconic acid; Pre-PMA, prephenyl mercapturic acid; PMA, phenyl mercapturic acid; HQS, hydroquinone sulfate.

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